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On the specificity of different methods for neutral detergent fiber and related problems

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Highlights

- Alternative NDF methods lack specificity by not yielding comparable results
- Variances for NDF from different feeds and analytical methods are heteroscedastic
- Normal and Beta distributions both apply to observed variations in NDF analyses

Abstract

We compared alternative methods for analyzing Neutral Detergent Fiber with the reference AOAC 2002.04 method for specificity. We reported the chemical composition of major nutrients for the matrixes of 19 feeds and one non-feed (sawdust). Two independent matrixes were from animal origin: meat and bone meal, and milk powder. All fiber residues were ash and blank-corrected to yield values comparable to the reference aNDFom obtained with the reflux in crucibles (Fibertec, method 1) or in Berzelius beakers without spouts (method 2). The alternative fiber methods were as follow: reflux in Berzelius beakers with spouts (method 3), the ANKOM pressurized filter

bag system (method 4), a non-pressurized filter bags (nonwoven tissue) immersion system (TECNAL, method 5), and micro-NDF digestion in crimp-sealed penicillin flasks deposited in an autoclave (method 6). The major nutrients were fractional mass proportions described as Beta-distributed variables. Assuming aNDFom as normally distributed because of negative fiber values recorded, an information-theoretic approach identified the relevance of the random effects of analyst (three analysts of the same laboratory) and their interactions with matrix, method, and matrix-method interaction as fixed effects. We also challenged the traditional homoscedastic assumption. The GLIMMIX procedure of SAS was used to fit the models. In sequence, we chose the model without all random effects except for the analyst-method-matrix interaction defined as the subject and a complete heteroscedastic structure with one variance estimate for each method-matrix group. We removed milk powder from the dataset to fit the same model by assuming that aNDFom/1000 was Beta-distributed, and Pearson residuals demonstrated a comparable fit to the normal assumption. Within the limits of the experimental error, the ADFom was entirely formed by Lignin (sa) for meat and bone meal and for powdered milk. We observed a significant method-matrix interaction for aNDFom. Therefore, effect slices for methods within matrix and vice-versa revealed several significant contrasts between reference and alternative methods for aNDFom. Methods 1 and 2 were not different. With powdered milk removed from the analysis, the fit under Beta-distributed aNDFom resulted in an increased number of significant contrasts; however, contrasts between methods 1 and 2 remained non-significant, regardless of the matrix analyzed. Significant contrasts demonstrated the lack of specificity of the alternative methods used to measure insoluble fiber compared with the reference methods. In terms of variability, within-laboratory variance for aNDFom corresponded to the method-matrix-analyst

component, and the variation introduced by different analysts in the same laboratory was negligible.

Keywords

Analytical Methods; Insoluble Fiber; Fiber Analysis; Fibrous Organic Matter; Heteroscedasticity; Repeatability

1. Introduction

Since the original, landmark publication by Van Soest and Wine (1967) regarding the determination of "Plant Cell-Wall Constituents" fifty years ago, food science has been transformed, with repercussions in agricultural, animal, nutritional, ecological, and even human health sciences (Chandler et al., 1980; Mongeau and Brassard, 1982; Van Soest, 1994; Mertens, 2003). Nonetheless, one of the most striking advancements of the detergent system was the division of food matter into two mutually exclusive fractions: an insoluble entity with a highly variable, non-uniform physicochemical and nutritional composition, and a chemically heterogeneous but soluble counterpart as an ideal, nutritionally uniform fraction with constant and highly predictable digestibility (Van Soest, 1967; Huhtanen et al., 2006). This achievement was possible because of the nutritionally oriented goal of the creators of the method, i.e., to obtain a "fibrous" residue low in nitrogen content that is not digestible by gastrointestinal-secreted enzymes of animals but utilizable to a variable extent by gut microbiota and that is also closely associated with indigestibility (Van Soest, 1963b; a; 1964; 1967; Van Soest and Wine, 1967). However, the senior creator and his colleagues eventually considered the original method obsolete (Van Soest et al., 1991). Because Van Soest and Wine (1967) created a method intended to isolate a specific insoluble fiber residue applicable to all feedstuffs, several modifications have been introduced and tested to solve problems of starch and protein contamination, lipid interferences, particle losses and filtration problems to

achieve desirable levels of specificity and reliability for the isolation of the intended chemical fraction (Ferreira et al., 1983; Van Soest et al., 1991; Udén, 2006; Ferreira and Mertens, 2007).

Although clearly relevant, the method that became known worldwide as neutral detergent fiber (NDF) did not receive an overall approval by the Association of Official Analytical Chemists (AOAC) immediately. The only method developed by Van Soest to reach that status was AOAC method 973.18, first action in 1973, which was designed to replace crude fiber with a fibrous residue recovered after acid detergent extraction and as a major preparatory step for the determination of lignin in H_2SO_4 (Van Soest, 1963b; 1973). However, only in 2002, AOAC approved the first action of a collaborative study by Mertens in an effort to standardize a widely applicable method to determine the insoluble fiber residue for all feeds. In that collaborative study, the measurand that presented the lowest inter-laboratory variability was the blank-corrected, organic fibrous residue obtained after ND extraction with additions of standardized α-amylase solution and anhydrous Na_2SO_3 (Mertens, 2002; 2003), known specifically as aNDFom. This acronym is used to link the standardized method to a proper terminology (Udén et al., 2005). A fundamental premise operates for this type of empirical analytical method: the isolated, specific analyte (measurand) is the result of the operational conditions of method execution. Thus, the isolated amount depends on reagents, equipment and glassware specifications, level of analyst expertise, and several other extraneous factors that might interact and affect the isolation of the chemical fraction of interest (Lucas, 1964; Horwitz et al., 1990; Mertens, 1996). However, researchers have been making several modifications of the NDF method based on the belief that deviations from the recommended procedure do not affect the results, i.e., do not affect the specificity and reliability of this empirical method. Such modifications are rarely documented, despite

the fact that they are usually accompanied by a "proper citation"; however, we did not address the reasons for that belief in this paper.

Currently, several methods used to determine the NDF residue are variants of the original procedure of Van Soest and Wine (1967). Conserved are the reagents, but the use of a heat-stable α-amylase and an 8 M urea solution for soaking starchy feeds are considered necessary additions; however, the use of anhydrous Na_2SO_3 may be regarded as optional (Van Soest et al., 1991). Among alternative methods are innovative technologies such as filter bag systems that solved filtration problems by abolishing the use of Gooch crucibles (Mertens, 1998; Vogel et al., 1999; Ferreira and Mertens, 2007), although at the expense of not keeping feed particles in continuous suspension as occurs in the reflux systems for crucibles or beakers (Goering and Van Soest, 1970; Undersander et al., 1993; Mertens, 1998). A micro-NDF method designed to establish the relationship between in vitro gas production and the NDF mass digested abolished the use of crucibles or beakers and reduced the amounts of samples and reagents but introduced the ND reflux of feed particles in crimp-sealed penicillin bottles deposited in an autoclave for NDF recovery (Pell and Schofield, 1993). All these methods present important documented methodological departures from the standardized procedures described in AOAC method 2002.04 (Mertens, 2002), which contribute to reduce the method reliability and compromise specificity in terms of the analyte intended to be isolated (Mertens, 1996). We adapted the aforementioned methods to recover a fibrous organic matter residue as a common target measurand with the goal of estimating within-laboratory variances and point and interval estimates to compare different methods (including the reference method), feed matrixes, and analysts.

2. Materials and Methods

We obtained samples from 19 materials representing feed matrixes and one sample of sawdust (high cellulose material matrix) to compare the performance of six methods used to quantify the insoluble fiber and to estimate the variability of operators, methods, and feeds. The materials presented a wide range of aNDFom (≈0–900 g/kg DM), determined on an ash-free, blank and DM-corrected basis (Mertens, 2002). The different matrixes also included materials with high fat content, high ash content, by-products, oilseeds, and samples of fresh and conserved forage as silage and hay of legumes and tropical and temperate grasses, in addition to matrixes from animal origin. The list of materials in all tables and results is presented in the following order: (1) ground yellow corn grain (*Zea mays* L.); (2) wet brewers' grain, mostly from barley (*Hordeum vulgare* L.); (3) whole soybean seeds (*Glycine max* (L.) Merr.); (4) cottonseed meal (*Gossypium* spp.), solvent extracted; (5) corn silage; (6) Tyfton 85 (*Cynodon* spp.), 45-day harvested hay; (7) alfalfa (*Medicago sativa* L.), 28-day harvested hay; (8) dried citrus pulp; (9) solvent-extracted soybean meal; (10) sorghum (*Sorghum bicolor* L.), ground grain; (11) wheat middlings; (12) elephant grass (*Pennissetum purpureum* Schum.), irrigated and harvested at 170 cm in height; (13) dried corn gluten feed; (14) corn husks; (15) sugar cane (*Saccharum officinarum* L.); (16) powdered milk; (17) soybean hulls; (18) meat and bone meal; (19) sawdust; and (20) ryegrass (*Lolium multiflorum* L.), as late cut hay.

2.1. Sample preparation

High moisture samples such as fresh and conserved forage and wet brewing bagasse were partially dried in a forced-air drying oven at 55 °C for 72 h. In sequence, coarse samples were quantitatively ground in a cutting mill to pass through a 5 mm screen, and a subsequent quartered aliquot was further quantitatively ground through a 1 mm screen to yield working samples that were stored in numbered plastic bottles to avoid bias from different analysts (Undersander et al., 1993).

2.2. Chemical composition

Only one analyst performed duplicate analysis of all material represented feed matrixes to avoid possible variations introduced by different analysts on the chemical composition that might have influenced NDF values, e.g., the DM content (Mertens, 2003). Therefore, prior to the fiber analyses, we obtained the total dry matter (DM, Undersander et al., 1993); crude protein (CP, AOAC method 2001.11; Thiex et al., 2002); ash (AOAC method 942.05; Undersander et al., 1993); crude fat (CF, AOAC method 2003.06; Thiex et al., 2003); and acid detergent fiber (ADFom) and lignin as Lignin (sa) according to AOAC method 973.18, after Möller (2009). We computed the organic matter (OM) content by difference. The analyst performed all weighing procedures to the nearest 0.1 mg in the laboratory using an analytical balance (model AY220; Shimadzu do Brasil LTDA, São Paulo, SP, Brazil) settled on an anti-vibration table. The ADFom (ash-free AD residue) and Lignin (sa) methods were basically followed, excepting that, at the time, the analyst had available for ADF analysis only Berzelius beakers (600 mL, high form) ***with spouts*** to use in the digestion units with condensers (model MA450/6; Marconi Equipamentos para Laboratórios LTDA, Piracicaba, SP, Brazil).

The total DM determination method outlined by Undersander et al. (1993) is the combination of AOAC method 967.03 and additional procedures recommended according to the analytical experience of those authors. We determined the ash content accordingly, because we used porcelain crucibles (AOAC method 942.05) with porcelain covers (Undersander et al., 1993). However, we used two types of muffle furnace: one with the temperature electronically programmable (model Q318M24; Quimis Aparelhos Científicos, Diadema, SP, Brazil), and the other analogic (model Q318D24; Quimis Aparelhos Científicos, Diadema, SP, Brazil) in which the temperatures were manually

increased to gradually reach 525 ± 15 °C, with the crucibles previously displaced in the furnaces.

The repeatability and reproducibility of the Kjeldahl CP method (AOAC 2001.11) are adequate for digestion in aluminum blocks using copper catalyst, K_2SO_4, H_2SO_4 and 1 g of feed sample into 250 mL borosilicate digestion tubes. Nevertheless, we used 100 mL micro-assay borosilicate tubes for digesting samples in two 40-probe aluminum blocks (model TE-040/25; Tecnal Equipamentos Científicos, Piracicaba, SP, Brazil); the tubes also fit to a specific distillation unit (TE-0363; Tecnal Equipamentos Científicos, Piracicaba, SP, Brazil). We proportionally adjusted the amounts of catalyst-salt mixture (one shallow, small porcelain spoon per tube yielded $\bar{x} = 1.003$ and SD $= 0.1049$ g, $n =$ 10 weighings) for 0.25 g samples and also sulfuric acid (5 mL per tube). The concentration of the NaOH neutralization solution used was 40% (w/w). We determined duplicate blanks per 40-probes run, and we used certified purity $NH_4H_2PO_4$ and Lysine-HCl to check for N-recovery of the distillation step and the entire method, respectively (Thiex et al., 2002).

The cellulose thimble used in the CF AOAC method 2003.06 is too expensive to discard after use. Therefore, we reused the thimbles, but we packed each feed sample using two sheets (10 cm side for concentrates and 13 cm side for roughages) of quantitative filter paper and deposited the packs inside the thimbles. In sequence, we performed the extraction with 175 mL of hexane (isomers mix, reagent grade) in an eight-probe solvent extraction unit (model MA491/8; Marconi Equipamentos para Laboratórios LTDA, Piracicaba, SP, Brazil) but immersed the thimble containing the packed sample for 30 minutes in the boiling solvent and washed the assembly for 50 minutes. We recorded the CF mass recovered in tared glass bottles. In spite of these modifications, we followed the other referenced recommendations (Thiex et al., 2003).

2.3. Fiber methods

Because the focus of this study were to estimate the variations of methods, analysts, and possibly their interacting effects, three analysts of the same laboratory independently performed all fiber methods used to quantify fiber in all material feed matrixes. Only one analyst was familiar with the official method, but no one had formal training on any one of the alternative methods. Therefore, they can be considered to have had at the time of the experiment low-to-mid experience in the analysis of feeds and fibrous foods. However, before the execution of the analyses, the analysts received a copy of the description of each analytical procedure and additional instructions to execute the tasks: no communications occurred about either the (1) methods performed or (2) results obtained. The analysts performed all weighing procedures to the nearest 0.1 mg using two analytical balances (models AY220 and AUY220; Shimadzu do Brasil LTDA, São Paulo, SP, Brazil), both settled on respective anti-vibration tables. Each analyst prepared and stored neutral detergent (ND) and amylase working solutions individually. The analysts prepared the ND solution for all methods according to Mertens (2002). The analysts shared each feed material in the same numbered-identified flask to minimize sample variation. Additionally, the analysts did not know the feed type; nonetheless, they received specific processing instructions about the requirement for pre-extraction with acetone to remove fat (for feeds with CF $\geq$ 50 g/kg DM) accordingly (feeds numbers 3, 16, and 18). Analysts also added anhydrous Na_2SO_3 for all methods.

2.3.1. Methods for reference fiber values (aNDFom)

The analysts performed the reference fiber methods (AOAC method 2002.04; Mertens, 2002) to recover aNDFom by refluxing the feed samples in crucibles (Fibertec) or in Berzelius beakers. The analysts followed the recommended methods and all details exactly to obtain an ash-free, DM and blank-corrected aNDFom measure. Fibertec™ 1020

digestion and 1021 cold extraction units (Foss, Suzhou, China) were used to prepare a fibrous residue filtered in Gooch crucibles (USP2, 26–28 mL, 40–90 μm pore size). Analysts also used a traditional reflux system, which was a digestion unit equipped with glass condensers (six-probes, model MA450/6; Marconi Equipamentos para Laboratórios LTDA, Piracicaba, SP, Brazil) to hold Berzelius beakers (600 mL, high form, without spouts), in which the prepared fibrous residue was recovered in Gooch crucibles (50 mL, high form, 40–60 μm pore size). The analysts tested all crucibles for filtration rates: 25 mL of dist. H_2O was filtered by gravity for 75 ± 30 s in crucibles used in the Fibertec system and for 180 ± 60 s for 50 mL of dist. H_2O in crucibles used in the beaker reflux system. All crucibles that did not pass the respective tests were discarded. The analysts also used a commercial heat-stable amylase (Novozymes Termamyl® 2X KNU-T/g; Tecnoglobo, Curitiba, PR, Brazil) to produce the working amylase solution according to the recommended standardization procedure. The Fibertec and the beaker reflux methods are henceforth named methods one and two, respectively. The analysts used a filter aid in the form of 8–12 g of washed and ashed beach sand for feed no. 18 (meat and bone meal) because of filtering difficulties. Reference fiber values were computed by accounting for ash and blank corrections and are expressed as average results to the nearest 1 g/kg DM, with error magnitudes (standard deviations) to the nearest 0.1 g/kg DM. Analysts also followed the recommended procedures for washing crucibles, such as reverse washing and sonicating, among other steps.

2.3.2. Refluxing in Berzelius beakers ***with spouts***

Several studies used Berzelius beakers with spouts to obtain an ND insoluble fiber residue. Therefore, we evaluated the reflux method by replacing beakers ***without*** spouts with beakers ***with spouts***. Analysts followed all of the other recommended procedures of method AOAC 2002.04 exactly. We ascribed number three to this method.

2.3.3. Pressurized filter bag method

Researchers have used the filter bag system under pressurization (ANKOM technology) because of the increased automation and performance advantages, particularly the greater number of samples that can be analyzed per run (Mertens, 1998). To evaluate this method, the three analysts performed the method independently using the 20 matrixes. They used model ANKOM^{220}, F57 filter bags and followed all details outlined in the NDF method 6 documentation, such as fat pre-extraction, collapsible desiccant pouch use, and addition of anhydrous Na_2SO_3 and FAA heat-stable alpha-amylase (Ankom® Technology, Macedon, NY, USA, www.ankom.com/sites/default/files/document-files/ Method 6 NDF A200.pdf) per 2 L of ND solution. The extraction unit capacity was 24 bags, including one blank. Because only 20 material feed matrixes were analyzed, analysts included four blanks. Additionally, the bags containing the fibrous residue and blanks were ash-corrected by depositing each bag in a porcelain crucible with cover and incinerating it in a muffle furnace at 500 °C for five h. Therefore, the blank-corrected fibrous organic matter (comparable aNDFom, g/kg DM) was computed as follows:

$$\text{aNDFom} = 1000\left(W_{br} - \left(W_b\bar{C}_1 - (\bar{W}_{cba} - \bar{W}_{cb})\right) - (W_{ca} - W_c)\right)/(DM_L W_s) \quad \text{Eq. (1)}$$

In which W_{br} is the post-extraction weight of the bag with NDF residue, W_b is the bag tare weight, and $\bar{C}_1$ is the blank bag correction as an average (four measurements per analyst per run) of post-extraction blank bag weights divided by the original bag tare weight. The parameter $\bar{W}_{cba}$ is the average weight of the porcelain crucibles plus ash of the post-extraction blank bag, and $\bar{W}_{cb}$ is the average tare weight of the crucibles (four measurements per analyst per run). The term W_{ca} is the weight of the porcelain crucible plus ash of the bag containing the fiber residue, and W_c is the porcelain crucible tare weight. DM_L and W_s are the proportions of laboratory dry matter (dimensionless) and

sample weight (g), respectively. The pressurized filter bag system corresponds, henceforth, to method number four.

2.3.4. Non-pressurized filter bag method

The analysts also used a non-pressurized filter bag system to assess the aNDFom in the material represented feed matrixes (method number five, including, e.g., the pre-extraction of fat-rich samples). The analysis was performed via a non-pressurized extraction unit container coupled with a glass condenser (model TE-149; Tecnal Equipamentos Científicos, Piracicaba, SP, Brazil) to avoid excessive water vapor loss from the ND solution. The extraction unit also had vertical agitation and could accommodate 30 bags simultaneously in eleven trays vertically distributed in a container that held 3 L of ND solution. Analysts deposited matrix samples in tared, heat-sealed squared filter bags (5 cm sides) of nonwoven tissue (100 g/m^2). Heat-stable amylase (FAA; ANKOM® Technology) and anhydrous Na_2SO_3 were added at the same proportions per sample, and other recommended procedures were followed in the pressurized filter bag method (method number four). Analysts computed the comparable aNDFom using Eq. (1). However, to complete the 30 bag capacity, six bags filled with grass forage samples were added to the 24 bags (20 feed samples and 4 blanks).

2.3.5. Micro-NDF method

Pell and Schofield (1993) suggested using an autoclave to assess the fibrous residue after incubating forage samples in vitro, which we named method number six. This procedure was used because of the advantages of processing many samples and using reduced amount of reagents per sample. We adapted the original method with two additions of 50 µL of amylase (FAA; Ankom® Technology). We used 50 mL glass penicillin flasks to hold 166 mg of anhydrous Na_2SO_3, 100 mg of sample (same proportion as used in method four), and 20 mL of ND solution. Analysts first added 50 µL of amylase before

extraction in the autoclave, closed the flasks with rubber stoppers, sealed them with aluminum crimp-seals, and mixed their contents manually. In sequence, each analyst deposited the sealed flasks in a previously heated autoclave (model Phoenix; Phoenix Luferco – Indústria e Comércio de Equipamentos Científicos, Araraquara, SP, Brazil) for independent runs. Each analyst performed the extraction under 0.2 kgf/cm^2 to maintain the internal temperature of the device at ca. 105 °C for one h. After extraction, the flasks were removed and their respective contents transferred quantitatively into previously tared Gooch crucibles (Fibertec users, 26–28 mL, 40–90 μm pore size) equipped with glass wool filter discs (A/E, 47 mm; Pall Life Science, New York, NY, USA). After the transfer and while the contents remained hot in the crucible, the analysts added a second 50 μL of the same amylase solution. Afterward, they followed the remaining procedures outlined by Mertens (2002), including the computation of the comparable blank-corrected aNDFom using Eq. (1). Fat-rich samples were pre-extracted by soaking and rinsing samples in the Gooch crucibles (Mertens, 2002) equipped with filter aid discs with 20–30 mL of acetone, and then the samples were transferred quantitatively to the penicillin flasks with the ND solution.

2.4. Statistical analyses

2.4.1. Chemical constituents

For the chemical composition of the material represented feed matrixes, proportions are presented as of the original material as is or as corrected to DM. Because these are continuous proportions, the probability density function (p.d.f.) most suitable to such variables is the Beta distribution (Mood et al., 1974; Ferrari and Cribari-Neto, 2004; Carlton and Devore, 2014; Stroup, 2015). Therefore, we estimated the chemical composition of the matrixes in terms of DM, OM, Ash, CF, CP, ADFom, and Lignin (sa) using the following stochastic model:

$$p_{ij} \sim \text{Beta}(\pi_{ij}, \phi) \quad \text{Eq. (2)}$$

$$\eta_{ij} = \text{logit}(p_{ij}) = \log(p_{ij}/(1 - p_{ij})) = \eta + \tau_i + e_{ij} \quad \text{Eq. (3)}$$

A general constant η is associated with the transformed mean (η_{ij}), which is linked by a logarithmic function (logit) to a linear predictor (Vonesh, 2012; Stroup, 2015). The expected values of the chemical constituents, that is, $\pi_{ij} \in (0, 1)$, are estimates based on the inverse "link" function as follows:

$$\text{E}[p_{ij}] = \pi_i = 1/(1 + \exp(-\hat{\eta}_i)) \quad \text{Eq. (4)}$$

In which $\hat{\eta}_i$ from Eq. (3) is the expected linear predictor $\text{E}[\eta_{ij}] = \hat{\eta} + \hat{\tau}_i$ and π_i is the fractional mass proportion of the expected chemical constituent. The observed $p_{ij} \in (0, 1)$ is the proportion corresponding to the chemical constituent (e.g., DM/1000, CP/1000, etc.) of the *i*-th feed (τ_i) obtained after the *j*-th determination or laboratory replicate (e_{ij}). The parameter $\phi > 0$ is a variance scale parameter that completes the variance of the Beta distribution, after Ferrari and Cribari-Neto (2004), as follows:

$$\text{V}[p_{ij}] = \pi_i\,(1 - \pi_i)/(1 + \phi) \quad \text{Eq. (5)}$$

We used the Restricted Pseudo-Likelihood Method (RSPL) of the GLIMMIX procedure of the SAS statistical software (SAS Studio, University Edition, SAS System Inc., Cary, NC, USA) to fit this model to the observed concentrations of the chemical constituents. One of the requested outputs ("ilink" function) of the GLIMMIX procedure is based on Eq. (4) and variances according to Eq. (5). We presented the final chemical constituent concentration as the product $1000\pi_i$ and the standard deviation of the estimate as $1000(\widehat{\text{V}}[p_{ij}])^{0.5}$ (expressed as g/kg as is for total DM and g/kg DM for other major nutrients).

2.4.2. Fiber methods

The analyst, material represented feed matrix, and method were effects listed in the basic statistical model. The matrixes and methods were levels of the fixed-effects,

whereas each of the three analysts was a random level of the analyst effect. Because some methods yielded negative fiber values for powdered milk (feed no. 16), the Beta distribution did not apply, because the observed proportions of fiber must belong to the open $(0, 1)$ interval. Therefore, we fitted Eq. (6) to the aNDFom (g/kg DM) values obtained from the different methods as follows:

$$y_{ijkl} = \mu + \tau_i + \alpha_j + \tau\alpha_{ij} + a_k + \tau a_{ik} + \alpha a_{jk} + \tau\alpha a_{ijk} + e_{ijkl} \quad \text{Eq. (6)}$$

In which y_{ijkl} is the aNDFom value from the *l*-th replicate performed by the *k*-th analyst (a_k) employing the *j*-th fiber method (α_j) on the *i*-th matrix (τ_i). The $\tau\alpha_{ij}$ interaction is also a fixed effect, whereas all other interaction effects that contain the random analyst effect are random, and e_{ijkl} is the residual. Because of its domain, the parameters of the Normal p.d.f. satisfied $-\infty < \text{mean} < \infty$ and variance > 0, and we assumed that the normal distribution might mimic the variability of the fiber data and therefore varied the specification of the mean $(\mathrm{E}[y_{ijkl}] = \mu_{ijk})$ and variance $(\mathrm{V}[y_{ijkl}] = \sigma_Y^2)$ according to the following equations:

$$\mathrm{E}[y_{ijkl}] = \mu_{ij} = \mu + \tau_i + \alpha_j + \tau\alpha_{ij} \quad \text{Eq. (7)}$$

$$\mathrm{E}[y_{ijkl}|a_k] = \mu_{ijk} = \mu + \tau_i + \alpha_j + \tau\alpha_{ij} + a_k \quad \text{Eq. (8)}$$

$$\mathrm{E}[y_{ijkl}|a_k, \tau a_{ik}] = \mu_{ijk} = \mu + \tau_i + \alpha_j + \tau\alpha_{ij} + a_k + \tau a_{ik} \quad \text{Eq. (9)}$$

$$\mathrm{E}[y_{ijkl}|a_k, \tau a_{ik}, \alpha a_{jk}] = \mu_{ijk} = \mu + \tau_i + \alpha_j + \tau\alpha_{ij} + a_k + \tau a_{ik} + \alpha a_{jk} \quad \text{Eq. (10)}$$

$$\mathrm{E}[y_{ijkl}|a_k, \tau a_{ik}, \alpha a_{jk}, \tau\alpha a_{ijk}] = \mu_{ijk} = \mu + \tau_i + \alpha_j + \tau\alpha_{ij} + a_k + \tau a_{ik} + \alpha a_{jk} + \tau\alpha a_{ijk} \quad \text{Eq. (11)}$$

Equation (7) is the expected mean (best linear unbiased estimate) in the absence of the random effects of analyst and all other random interaction effects. Equations (8)–(11) are the best linear unbiased predictors of the mean in the presence of the random interaction

effects. Additionally, we challenged the homoscedasticity assumption by fitting the following variance functions:

$\sigma_Y^2 = \sigma^2$ Eq. (12)

$\sigma_Y^2 = \sigma_i^2$ Eq. (13)

$\sigma_Y^2 = \sigma_j^2$ Eq. (14)

$\sigma_Y^2 = \sigma_{ij}^2$ Eq. (15)

Equation (12) represents the traditional homoscedastic assumption, and Eqns. (13)–(15) correspond to heterogeneous variances by matrixes (σ_i^2), methods $\left(\sigma_j^2\right)$ and by $\tau\alpha_{ij}$ interaction groups $\left(\sigma_{ij}^2\right)$. We used the GLIMMIX procedure to fit Eq. (6) and all models used to represent the mean (Eqns. 7–11) and variance functions (Eqns. 12–15) accordingly using the maximum pseudo-likelihood estimation method (MSPL). The nonlinear optimization option chosen was the Newton-Raphson with ridging (TECHNIQUE=NRRIDG). To evaluate the goodness-of-fit of the different models fitted to the aNDFom variable, we recorded the Akaike information criterion (Akaike, 1974) corrected for small samples as $AICc_r$ (Sugiura, 1978; Cavanaugh, 1997). We also computed the $AICc_r$-derived measures, namely, Akaike differences or Δ_r, model probabilities (w_r), and evidence ratios (ER_r), for each one of the r-th model combinations fitted (Burnham and Anderson, 2004). We discarded models whose fits did not converge. Models that presented non-positive definite covariance matrices at convergence were rerun, but without the zeroed variance estimates to solve the problem of non-positive definiteness (Littell et al., 2006). Based on the information criteria computed for the fitted model combinations, we chose the model that presented the lowest level of uncertainty (Table 1), i.e., $1 - w_r \cong 0$ (Buckland et al., 1997; Burnham and Anderson, 2004; 2014). In sequence, we fitted the chosen model using the restricted pseudo-likelihood estimation

method (RSPL), which is the default estimation in the GLIMMIX procedure. After checking the significance of the fixed interaction effect, we requested tests on effect slices of feeds within method, methods within feeds and pairwise multiple range tests. The pairwise tests requested using GLIMMIX were the result of a simulation adjustment on the *P*-values of the contrasts, which also prevents the inflation of the type I error rate due to the many multiple comparisons performed (Littell et al., 2006). Because analysts of the same laboratory conducted all fiber methods to predict the fiber values of all matrixes and to avoid false positive findings (Johnson, 2013), we adopted two significance level ranges regarding the type I error rate: $0.001 \leq P < 0.01$ for appreciable evidence of an effect; and $P < 0.001$ for strong evidence of an effect. Afterward, we removed the powdered milk (feed no. 16) to estimate the magnitude of the same effects of the chosen model by fitting it to aNDFom/1000 proportion as a Beta-distributed variable using the same GLIMMIX procedure. Because results for major chemical constituents are reported as g/kg, we reported means of DM, CP, Ash, CF, ADFom, Lignin (sa), and aNDFom to the nearest 1 g/kg, which corresponds to the literature 0.1% basis for major nutrients, and standard deviations to the nearest 0.1 g/kg (Horwitz et al., 1990).

2.4.3. Overall evaluation of fiber methods

The chosen model (Normal p.d.f., $w_r \cong 1$) was used to estimate the least squares means for each method by matrix interaction (Eq. 7). Additionally, the most suited model was the one with heterogeneous variances computed for each matrix by method interaction (Eq. 15). Therefore, the variance of analysts was not important to represent the data, and the combination of Eq. (7) with Eq. (15) predicted means and variances after assuming each $\tau\alpha a_{ijk}$ as subjects (Table 1). The square roots of the variances of the least squares means based on this model were therefore representations of the within-laboratory variation (repeatability), and then, we named the estimated standard

deviations as $SD_{r_{ij}}$ and computed the relative standard deviation (%) as $RSD_{r_{ij}} = 100\,SD_{r_{ij}}/\mu_{ij}$, according to Horwitz (1982a). Mertens (2002) coordinated the first collaborative study to evaluate the neutral detergent fiber method at the time of this research, therefore the number of studies, i.e., $n_s = 1$. We computed the expected among-laboratory relative standard deviation ($PRSD_R$) and its standard error (SE_{PRSD_R}), which are measures of reproducibility (Horwitz, 1982a; 1982b; AOAC, 2016), based on our estimated least squares means as follow:

$$PRSD_R = 2^{(1-0.5\log_{10}(\mu_{ij}/1000))} \quad \text{Eq. (16)}$$

$$SE_{PRSD_R} = PRSD_R((1 + 2PRSD_R^2)/(2n_s))^{0.5} \quad \text{Eq. (17)}$$

The upper limit of the 95% confidence interval is approximately

$$PRSD_R + 2SE_{PRSD_R} \text{ (Horwitz, 1982a)} \quad \text{Eq. (18)}$$

We computed RSD_R values that yielded a constant Horwitz ratio (HORRAT) limit for an acceptable method as

$$RSD_{Rc} = 2PRSD_R. \quad \text{Eq. (19)}$$

One must remember that our computations yielded a heteroscedastic variance pattern, different from the original Horwitz estimation assumptions of normality and homoscedastic and independent normal errors among studies, which resulted in the approximately predicted within-laboratory relative error as follows (Horwitz, 1982a; 1982b; AOAC, 2016):

$$PRSD_r = 1.3(\mu_{ij}/1000)^{-0.1505}. \quad \text{Eq. (20)}$$

Equations (16)–(20) were applied to the chemical composition of the matrixes estimated under the Beta distribution, i.e., Eqns. (2)–(5).

We considered reference fiber values the estimated means for each matrix for both methods nos. 1 and 2. Therefore, with the help of the model evaluation system (MES,

https://nutritionmodels.tamu.edu/models/mes/), which is based on statistical measures of model accuracy and precision (Tedeschi, 2006), we compared the predicted means of the matrixes obtained for each alternative method of fiber analysis (respectively ascribed to X in MES) with the expected respective reference values (ascribed to Y in MES). A summary table containing the measures of accuracy and precision for each method compared with the reference ones is an output of the system. The output contains the general mean ($\bar{x}$), standard deviation ($\hat{\sigma}$), median ($\tilde{x}$), R^2, mean squared error of prediction of the alternative method (MSEP), mean bias of the alternative method (MB), method efficiency factor (MEF), coefficient of determination (CD) of the method, and the bias correction factor (C_b) as a component of the concordance correlation coefficient ($\hat{\rho}_c$). Additionally, we also requested as an MES output the results of the joint hypotheses tests for the intercept (θ_0) and slope (θ_1) of the regression of Y on X as $H_0^{(m)}$: $[\theta_0 \quad \theta_1] = [0 \quad 1]$ for $m = 1$, tested according to Mayer et al. (1994), and for $m = 2$, tested according to Dent and Blackie (1979).

3. Results

The effect of feed matrix was highly significant for all major fractions of the chemical composition (Table 2). The residual variances for feed matrixes were not homogeneous, because variances of Beta-distributed compositional data were dependent on the expected mean of the chemical component, as shown by Eq. (4). Of note, ADFom was entirely composed of Lignin (sa) in meat and bone meal (matrix no. 18). Within the limits of the error, the concentrations of ADFom and Lignin (sa) in the powdered milk (matrix no. 16) were also equivalent. The observed RSD_r decreased, as expected, as overall tendencies toward the analytes presented in larger mean concentrations. Analytes such as CF, ADFom, Lignin, Ash, and CP were highly variable at low concentrations. Among

these analytes, only 28 of the 120-recorded RSD_R were below the predicted RSD_{Rc} (Eq. (19)), which would yield a reproducibility HORRAT = 2 (Figure 1g).

As we described previously in sections 2.4.2 and 2.4.3, the AICc was smallest for Eq. (7) fitted to aNDFom values (under the normal assumption), without random effects other than $\tau\alpha a_{ijk}$ and e_{ijkl}, in which $\tau\alpha a_{ijk}$ was the subject effect, e_{ijkl} was suppressed, and heterogeneous variances for each material × method interacting group modeled as Eq. (15). This model combination yielded the highest model probability. Therefore, the level of uncertainty was almost nil in this choice of model combination when compared with the other different model combinations that were evaluated (Table 1). The chosen model (Eqns. (7) and (15)) detected many significant effect slices for methods within matrixes and significance for all effect slices of matrixes within each method (Table 3). The inclusion of all random factors as in Eq. (9) combined with Eqns. (12)–(15) did not improve the representation of the observed data (Table 1). This lack of improvement in the quality of fit of different mixed models was also revealed by the patterns of Pearson residuals (Figure 1, panels (a) and (b) *vs.* panels (c) and (d)). Despite its negligible model probability compared with the chosen model previously described (Table 1), we present, just for the record, the fit (RSPL estimation) of the traditional normal, independent and identically distributed errors (Figure 1, panels (c) and (d)) represented by the combination of Eq. (9) with Eq. (12) yielded the following variance estimates for the random effects: $\hat{\sigma}_a^2 = 29.4\ (0, 2.61)$; $\hat{\sigma}_{\tau a}^2 = 85.9\ (0.11, 0.45)$; $\hat{\sigma}_{\alpha a}^2 = 129.6\ (0.14, 1.03)$; $\hat{\sigma}_{\tau\alpha a}^2 = 104.8\ (0.12, 0.48)$; and $\hat{\sigma}^2 = 376.5 \pm 24.28$. These parentheses contain the 95% confidence bounds obtained by profile likelihood for the respective ratios of each variance component to the residual variance. A standard error accompanies the common residual variance.

In spite of the possible significant contrasts for methods within the respective matrixes nos. 2, 8, 16, and 18 (Table 3). A significant contrast was detected between methods nos. 2 and 4 for matrix no. 12 (Table 4). Method no. 4 yielded different estimates from other methods, including the reference ones, for several matrixes, and at least for one matrix (no. 3), method no. 3 differed from reference method no. 2 (Table 4). The reference fiber methods did not yield different results for all material matrixes analyzed (contrast 1-2) under the normal p.d.f. assumption. Nonetheless, there was also no evidence that the pairwise contrasts 1-3, 1-5, 1-6, 2-5, 2-6, and 3-6 differed from zero for all matrixes. Therefore, fibrous residues obtained with method no. 1 were not statistically different from those obtained with methods 3, 5, and 6, regardless of the matrix, but methods 5 and 6 did not yield comparable results. The fibrous contents obtained with reference method 2 were comparable to those obtained according to methods 5 and 6. Despite the use of Berzelius beakers with spouts in contrast to the use of crimp-sealed penicillin flasks, methods 3 and 6 yielded comparable results, regardless of the matrix.

Several results were above the ideal RSD_R limit (see section 2.4.3), as the dotted line in figure 1h reveals, despite the fact that RSD_R reproducibility estimators came from assumptions different from the ones assumed for this study. Indeed, several results were above the $2/3\ RSD_R$ limit for expected repeatability. Therefore, method no. 1 was highly variable in our laboratory for six feeds; method no. 2 was highly variable for 7 feeds; method 3 for five feeds; method 4 for six feeds (not counted, feed 16); method no. 5 for 9 feeds; and method no. 6 for 7 feeds (Table 3). All methods were below the expected RSD_R limit for roughages, namely, matrixes nos. 5, 6, 7, 12, 14, 15, 17, and 20; the same was true for the lignocellulosic material (sawdust, Table 3). The computed $RSD_{r_{1,1}}$ (method 1 applied to feed 1) was the only relative variability below the expected RSD_R limit for aNDFom of corn grain, whereas the computed $RSD_{r_{2,9}}$ was the only one below the

expected limit of the reference method for soybean meal. Matrixes nos. 3, 10, and 16 were highly variable compared with the expected RSD_R limit of the reference aNDFom method (Table 3). When methods 1 and 2 were combined and the estimable functions for the least squares mean of ground corn computed from the chosen model (Eqns. (7) and (13)), the following estimates were obtained: aNDFom $= 102 \pm 7.4$ g/kg DM, a 99% confidence interval of $(95, 109)$, an $RSD_r = 7.25\%$, and an expected limit for $RSD_R = 5.64\%$. Therefore, the joint analysis revealed that the repeatability was not satisfactory when the values 2 and 1.3 were considered the threshold limits for the HORRAT reproducibility and repeatability, respectively. The joint mean value for the reference methods 1 and 2 for concentrates was 229 ± 17.3 g/kg DM, with a repeatability $RSD_r = 7.57\%$ and an expected $RSD_R = 4.99\%$. For roughages, the joint aNDFom mean was 660 ± 13.3 g/kg DM, with an $RSD_r = 2.02\%$ and an expected limit for $RSD_R = 4.26\%$. The joint aNDFom mean for all matrixes was 427 ± 15.5 g/kg DM, with an $RSD_r = 3.63\%$ and a limit $RSD_R = 4.55\%$. Therefore, only concentrate feeds evaluated separately from other matrixes did not strictly follow the quality figures used for inter- and intra-laboratory performance evaluation.

Despite the lack of evidence in favor of contrasts 1-5 and 2-5, the highly variable method no. 5 yielded biased values that did not match the unity line with aNDFom means (Tables 3, 4 and 5). Method no. 5 presented a general overestimation bias (Table 5 and Figure 2e, f), whereas method no. 4 presented underestimated biased results, as revealed by the MB values and rejection of both null hypotheses tested for adherence to the unity line (Table 5 and Figure 2c, d). Nonetheless, correcting linear regression equations (also MES outputs) can be used to determine the expected reference value for a given matrix based on the ANKOM procedure: aNDFom (method 1) $= 12(\pm 6.9) + 1.01(\pm 0.014) \times aNDFom$ (method 4), $r^2 = 0.997$; and aNDFom (method 2) $= 11(\pm 4.3) +$

$1.02(\pm 0.009) \times aNDFom$ (method 4), $r^2 = 0.999$. These significant intercepts are indicators that method 4 produced constantly biased (Figure 1c, d). Although we did not reject both null hypotheses for coincidence with the unity line for means of methods one and two compared with that of the alternative method six, both MSEPs of method six were more than sixfold greater than the MSEP of the reference methods (Table 5 and Figure 2g, h). The MES values confirmed the absence of evidence for overall differences between methods nos. 1 and 3, as revealed by the accuracy measures MB, C_b and $\hat{\rho}_C$ (Table 5 and Figure 2a). The same occurred for methods nos. 2 and 3 (Table 5 and Figure 2b); nonetheless, the statistical model represented by the combination of Eqns. (7) and (15) was more sensitive in detecting contrasting differences between methods 2 and 3 for at least one matrix (Table 4). The MES accuracy and precision outputs regarding the comparison of methods nos. 1 and 2 confirmed the previous statistical results that the methods yielded comparable results (Tables 4 and 5).

With the exclusion of feed no. 16, the combined model formed by Eqns. (7) and (15) was fitted to the aNDFom/1000 as a Beta-distributed proportion. The plot of the observed against predicted values (Figure 1e) and the well-distributed Pearson residual pattern (Figure 1f) were additional indicators of a reasonable fit of the Beta-distributed assumption compared with the model fitted under the normal assumption (Figure 1a, b). Least squares means, values of $SD_{r_{ij}}$ and *P*-values based on the Beta p.d.f. did not altered the conclusions reached previously with the normally distributed errors and heterogeneous variances model (Table 6 *vs.* Table 3). Nevertheless, the Beta-distributed model fit increased the number of significant contrasts (Table 7), because the amplitudes of the confidence intervals generated for contrasts based on Beta p.d.f. were more precise (not shown). The exclusion of feed no. 16 and the Beta-distributed assumption favored the detection of significant pairwise contrasts 1-5, 1-6, 2-5, 2-6, and 3-6 for at least one

matrix, whereas only contrasts 1-2 and 1-3 remained with no evidence of statistical difference regardless of the matrix analyzed (Table 7). The contrast 5-6, which was significant under normality and with feed no. 16 included, under the assumption of a Beta distribution and with this matrix excluded, became non-significant (Table 7), regardless of the matrix.

The SD estimates of NDS values were the same as those obtained for aNDFom estimates under normality assumption and heterogeneity of variances (Tables 3 and 8). Therefore, *P*-values (Table 8) and adjusted *P*-values (not shown) were equal to those estimated for different matrix-method interacting groups (Tables 3 and 4).

4. Discussion

Our results confirmed that methods nos. 1 and 2 yielded comparable results, regardless of the feed analyzed; thus, the two methods presented the same applicability (Horwitz, 1982b). For some feeds, the reported standard deviations for methods 1 and 2 were above the limits set by the Horwitz equation. Our perception was that one of the crucial steps of the reference method is the standardization of the working amylase solution; nonetheless, poor performance of other steps could also contribute to both inaccurate and imprecise results (Mertens, 2007). However, note that the reported repeatability or within-laboratory variation, as obtained in our study, was not directly comparable with reproducibility or among-laboratory variation; actually, repeatability is approximately 1/2 to 2/3 the reproducibility based on assumptions of independent and identically distributed normal errors (Horwitz, 1982a). Different from collaborative studies, the standard deviations reported here were heterogeneous, that is, we obtained one variance estimate for each matrix by method group (Eq. (15), Tables 3 and 6), and we performed the comparison with $PRSD_R$ and $PRSD_r$ only to provide a reference basis for the readers. However, the fit of a model with all fixed and random factors included under

the assumption of homoscedasticity had no support after an evaluation performed according to the information-theoretic approach (Burnham and Anderson, 2014). In studies performed to estimate the among- and within-laboratory variation, the effects of different days or runs are considered within the limits of the statistical error and analyst effect is not included (Mertens, 2003). Our findings appear to corroborate that postulate, because the inclusion of the random analyst effect and its interactions with fixed effects did not improve the model probability (Table 1). As a point of emphasis, Eqns. (16)–(20) demonstrated the dependency of SD_r and SD_R on the concentration $0.001\,\mu_{ij}$ (Horwitz, 1982a; 1982b; Thompson, 1999). However, this dependency might be greater not only because of the heteroscedasticity described by Eq. (15) under the normal assumption but also by the additional dependency of the variance on scale as described by Eq. (5), Eq. (15), and Eq. (7) to complete the stochastic model utilized to summarize the variation in our data as a Beta-distributed aNDFom concentration. Equation (16) has been criticized for the large intervals of the predictions that are generated, which dictate that the performance of a given method cannot be predicted with any reasonable certainty. Critics argue that analyte type and concentration, improvements of equipment and analytical techniques over time, and different methods for the same analyte constrain the predictive power of the Horwitz equation (Linsinger and Josephs, 2006). Nevertheless, the laboratory manager can use Eq. (16) to cross-check results and to compare them to uncertainty estimates derived from relevant collaborative trials (Thompson, 2007).

The use of Berzelius ***beakers with spouts*** causing a natural loss of water from the boiling ND solution is a reasonable expectation. Nonetheless, Barbosa et al. (2015) found that both volume and concentration of pure detergent solution were relatively unaltered after boiling for one hour in beakers with spouts. However, pure analytes do not behave the same when added artificially compared with natural occurrence in complex matrixes

(Horwitz, 1982a); therefore, this aspect might also be applicable to solutions. Of note, Berzelius beakers "without spouts" in the original NDF, ADF, and Lignin reported methods were not mentioned (Van Soest, 1963a; b; 1965; Van Soest and Wine, 1967; Van Soest, 1973). Nonetheless, in USDA agriculture handbook 379, the use of "a. Beakers, Berzelius without spout, 600-ml. capacity" is specifically mentioned, in addition to the recommendation for use of coarse-porosity fritted glass crucibles (Goering and Van Soest, 1970). Mertens (2002) not only emphasized the necessity of using ***beakers without spouts*** and coarse-porosity crucibles (40–60 μm of pore size) for aNDFom determination in the reflux apparatus but also provided instruction on how crucibles must be cleaned and tested for filtration rates. The use of Berzelius beakers without spouts, blank correction, and criteria for cleaning and testing crucibles for filtration rates were also maintained after a collaborative study for ADFom and Lignin (sa) (Möller, 2009). In this regard, the condensers of the Fibertec did not cover the open extremity of the crucible-attachable digestion tubes. This open-ended tube might be one cause, inter alia, why we did not detect differences between methods nos. 1 and 3; nonetheless, our results indicated that fiber values from method no. 3 did not behave the same as the reference ones obtained with method two, which compromised the applicability and specificity of method three. In spite of the lack of specificity, the use of Berzelius beakers with spouts is recurrent in NDF analysis (Vieira et al., 1997; Malafaia et al., 1999; Carvalho et al., 2014; Barbosa et al., 2015; Processi et al., 2016).

The ANKOM method no. 4 has high practicability by increasing the number of determinations per run (24 simultaneous samples), reducing analyst time costs and increasing productivity through automation and reducing manipulation steps (Mertens, 1998; Vogel et al., 1999; Mertens, 2007). Nonetheless, the conditions within the digestion chamber are not uniform vertically, which results in gradually different aNDF values from

top to bottom trays; additionally, an interaction likely occurs between ANKOM filter bag and crucibles for at least fatty matrixes (Mertens, 1998). The addition of α-amylase for running the ANKOM analysis removes starch contamination and yields comparable results to crucibles in terms of aNDF for corn silage (Ferreira and Mertens, 2007); however, we found significant contrasts between methods two and four for the same forage (Tables 4 and 7). Vogel et al. (1999) reported that the NDF values obtained with the ANKOM method are systematically lower than those measured in crucibles without amylase. Our modifications on the ANKOM method yielded an amylase-treated, blank-corrected, fibrous organic matter residue systematically lower than that of the reference methods nos. 1 and 2, regardless of the feed matrix. The loss of very fine fibrous particles from the filter bags might explain part of that systematic reduction in the fibrous values obtained with method no. 4 (Vogel et al., 1999). Although regression analysis is not recommended to empirically evaluate model predictions (Mitchell, 1997), knowledge of a probable systematic and constant bias may be used for calibration based on regressing reference aNDFom values over ANKOM fibrous values. In our results, the variability of method no. 4 was high for feed matrixes 1, 3, 4, 9, 10, and 18, regardless of the analyst who performed the method. Among the contrasts reported in tables 4 and 7, all pairwise contrasts between methods 1 *vs.* 4 and 2 *vs.* 4 yielded estimates significantly greater than zero (data not shown), which also demonstrated that the ANKOM filter bag method underestimated the reference aNDFom values.

The non-pressurized system that uses filter bags made from nonwoven tissue (method no. 5) also yielded biased results; however, the bias was not constant as revealed by figures 1e and 1f. For method no. 5, fibrous residues were overpredicted at small and slightly underpredicted at large reference aNDFom concentrations. Method no. 5 did not yield acceptable relative standard deviations for several feed matrixes. Additionally,

based on the Beta distribution, method no. 5 lacked applicability, as defined by Horwitz (1982b), because some pairwise contrasts between methods 1 or 2 *vs.* method 5 were significantly different from zero for some feed matrixes (Table 7). Method no. 5 also did not perform equally to method no. 6: some pairwise contrasts were statistically different from zero, regardless of the distribution (Tables 4 and 7). Therefore, the specificities of methods 4 and 5 were different from one another and were different from the common specificity of methods 1 and 2; for specificity as a criterion, the intended meaning was that the method isolated the analytical fraction intended to represent the desired measurand (Horwitz, 1982a).

The micro-NDF method (no. 6) has been used for some time as an alternative to the reflux of samples in beakers (Pell and Schofield, 1993). Similar to the ANKOM method, the primary advantage of method no. 6 is the superior productivity. Analysts can accommodate several flasks inside the equipment, depending on the chamber capacity; moreover, laboratories often have autoclaves (Senger et al., 2008). Originally, glass filter fibers replaced fritted Gooch crucibles (Pell and Schofield, 1993). In fact, filter papers are alternatives for the recovery of fibrous residues (Van Soest et al., 1991; Udén, 2006). However, for comparison purposes, we used crucibles as in the reflux in beakers (Mertens, 2002; Barbosa et al., 2015) and computed the fibrous organic matter corrected for blanks as in Eq. (1). Graphically (Figures 2g and 2h), the behavior of the fibrous residues obtained with the autoclave followed tendencies similar to those reported by Barbosa et al. (2015), that is, overprediction for small and agreement for large aNDFom values estimated by the reference methods. Nevertheless, although the general hypotheses for the estimated least squares means, i.e., $H_0^{(1)}$: $[\theta_0 \quad \theta_1] = [0 \quad 1]$ (Mayer et al., 1994) and $H_0^{(2)}$: $[\theta_0 \quad \theta_1] = [0 \quad 1]$ (Dent and Blackie, 1979), were not rejected (Table 5), method no. 6 yielded fibrous values without the same applicability and analytic

specificity to all matrixes as reference aNDFom values, as revealed by pairwise comparisons when powdered milk (feed no. 16) was removed from the overall analysis (Table 7). The concentrations of aNDFom for the powdered milk obtained with reference methods nos. 1 and 2 were below the limits of detection for gravimetric analyses, because weighing such small residues does not allow reliable inferences (Horwitz et al., 1990). In fact, the reference method is applicable to feed matrixes ranging in aNDFom concentrations from 15 to 1000 g/kg, and when the analyte concentration is lower than 10% of the test portion, the reproducibility of gravimetric methods is 2 to 3-fold the $PRSD_R$ (Mertens, 2002).

4.1. *Conclusions*

We demonstrated that heteroscedastic variances are very likely for describing the variability observed for the isolation of fibrous residues of different feed matrixes by alternative methods when compared with the standard reference ones. Additionally, different analysts apparently do not inflate the heterogeneous within-laboratory residual variation; in fact, the analyst effect may be an intrinsic part of matrix-method variability. At this point, we resort to the Codex Alimentarius to associate specificity of a measurand determined with an empirical analytical method to understand the meaning of a reference value: "… a value that can only be arrived at in terms of the method per se and serves by definition as the only method for establishing the accepted value of the item measured" (Codex Alimentarius Comission, 2007). Therefore, any single modification of the recommended procedure for aNDFom determination, even the smallest one, can affect both specificity and reliability of the results. Fiber figures obtained by a modified method cannot be represented by the same acronym used for the reference value without a detailed explanation of the modifications adopted (Udén et al., 2005), and any comparison about fiber values must be performed with caution because departures from the

recommended procedures for the empirical quantification of aNDFom could affect the specificity of the resulting values, which compromises the inference space of fiber-related results.

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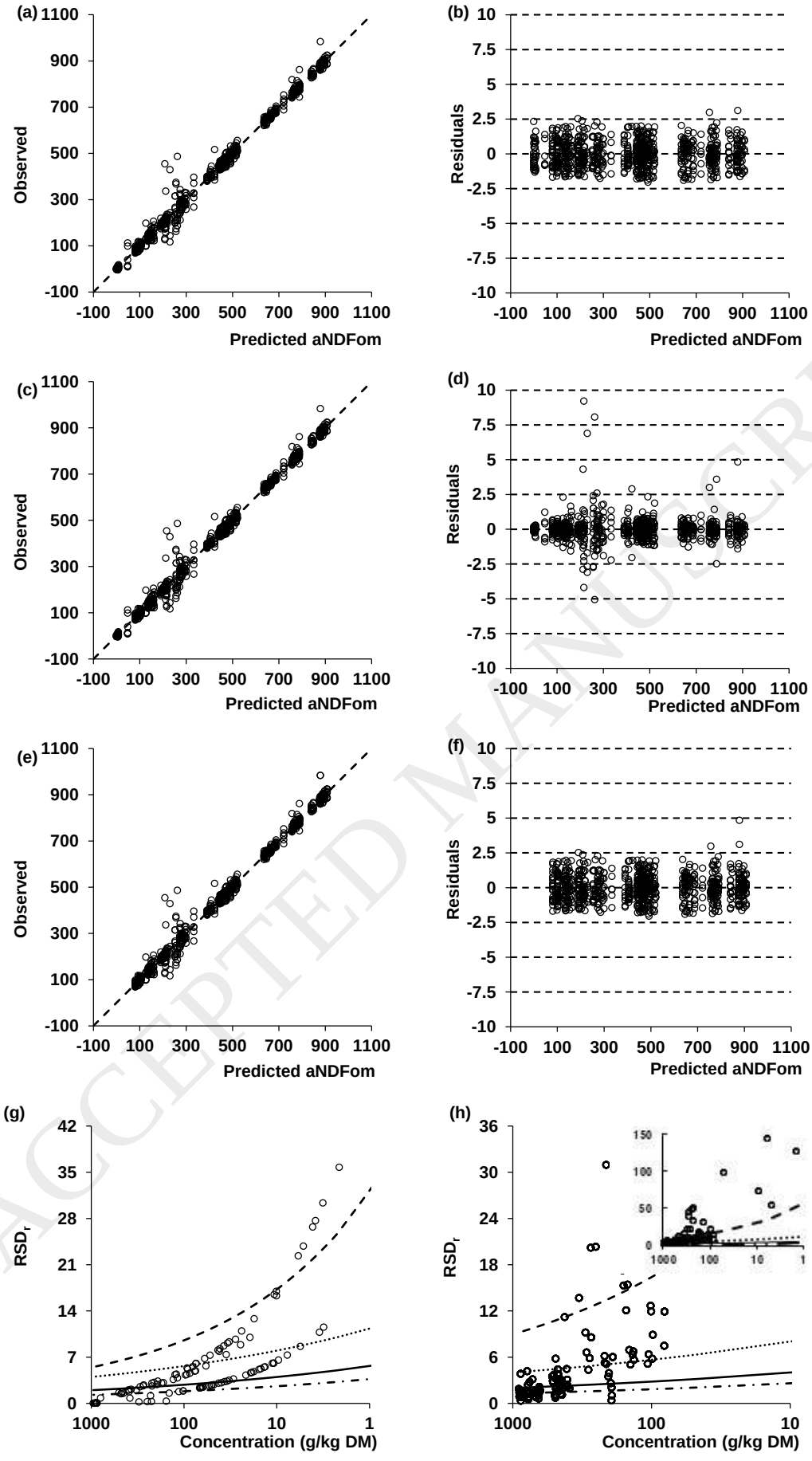

Figure 1. Plots of the observed (circles) against predicted aNDFom (g/kg DM) according to the model with normal heterogeneous variances, which was most suited fit to represent the data (a). On panel (c) is depicted the fit under normality of the mixed model with random effects of analyst and its doubles and triple interactions with the fixed factors method and feed matrix, and on panel (e) is depicted the most suited model fitted under the assumption of a Beta distribution (without feed matrix no. 16). On panels (b), (d), and (f) are the respective plots of the Pearson residuals generated after fitting models shown on (a), (c), and (e). On (g) and (h) are shown the within-laboratory relative standard deviations (RSD_r, circles) for chemical composition and for aNDFom based on the fit of panel (a) model (complete set in the upper detail). Panels (g) and (h) contain solid and dashed-dotted lines as the predicted Horwitz RSD among-laboratories ($PRSD_R$) and $2/3PRSD_R$, respectively, the dashed line is the expected upper 95% confidence limit for $PRSD_R$, and the dotted line is the RSD_{Rc} limit for a Horwitz ratio equal to two.

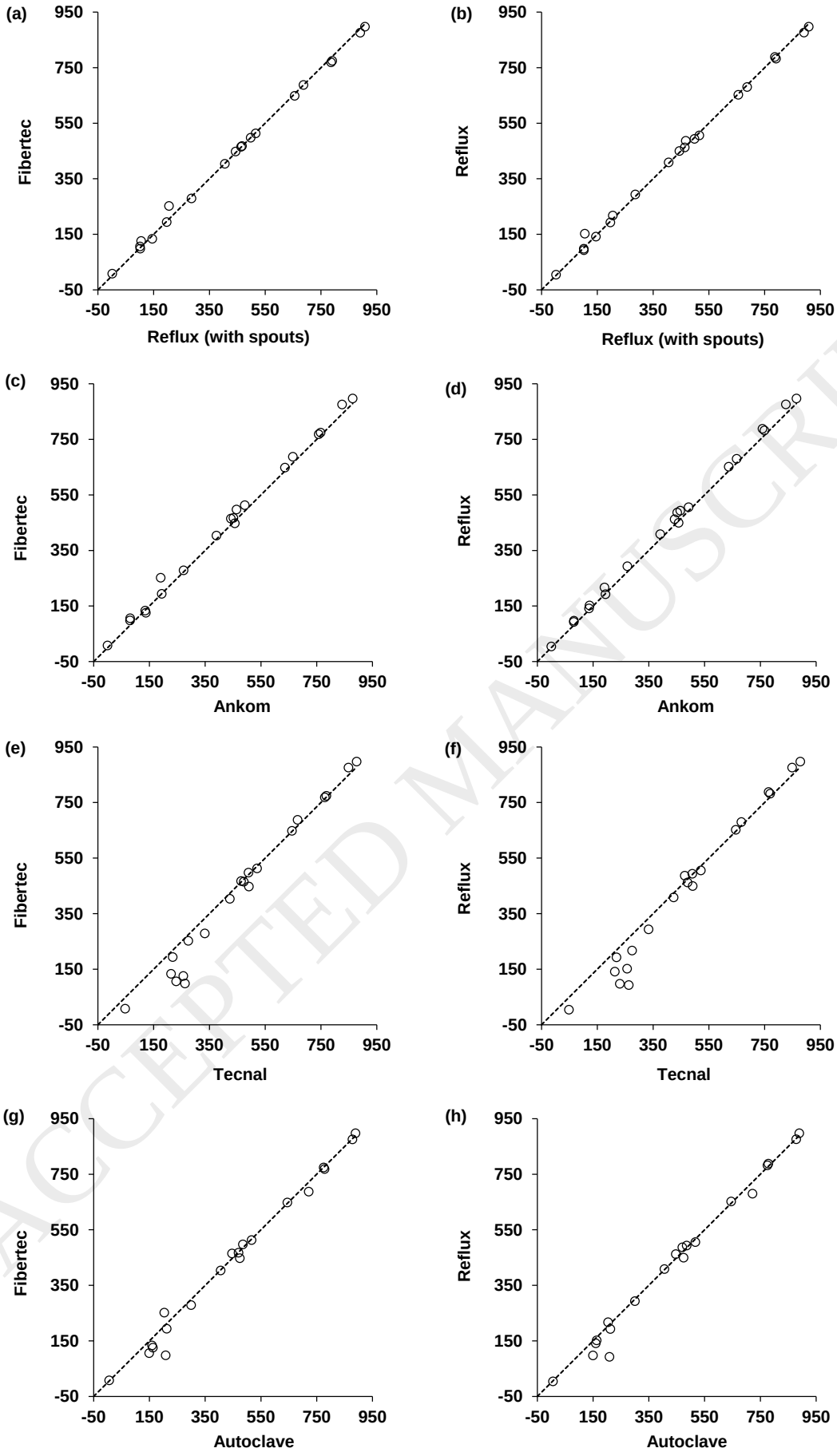

Figure 2. Comparison of reference Fibertec (reflux in crucibles) and Reflux (in beakers without spouts) methods (Y-axis) and the other alternative methods used to measure fiber values (X-axis), that is, reflux in beakers with spouts (panels a and b), pressurized Ankom method (panels c and d), non-pressurized Tecnal method (panels e and f), and the autoclave method (panels g and h). The circles are the correspondent coordinates of the predicted pairs of means for each method, and the dashed lines correspond to the unity lines (Y = X).

Table 1. Computed information criteria for combinations of the models containing all fixed effects with no or additional random factors (a_k, τa_{ik}, αa_{jk}, and $\tau\alpha a_{ijk}$) to the error term included, and different variance assumptions represented by σ_Y^2

Model: Random factors included	σ_Y^2[a]	$-2\ell^*$[b]	$AICc_r$[b]	Δ_r[b]	w_r[b]	ER_r[b]
None	σ^2	7794	8076	948	$\cong 0$	10^{205}
a_k	σ^2	7752	8037	909	$\cong 0$	10^{197}
$a_k, \tau a_{ik}$	σ^2	7724	8011	883	$\cong 0$	10^{191}
$a_k, \alpha a_{jk}$	σ^2	7676	7963	835	$\cong 0$	10^{181}
$a_k, \tau a_{ik}, \alpha a_{jk}$	σ^2	7634	7925	796	$\cong 0$	10^{172}
$a_k, \tau a_{ik}, \alpha a_{jk}, \tau\alpha a_{ijk}$	σ^2	7634	7927	799	$\cong 0$	10^{173}
sub $= \tau\alpha a_{ijk}$[c]	σ_i^2	7190	7525	397	10^{-87}	10^{86}
sub $= \tau\alpha a_{ijk}$	σ_j^2	7281	7577	449	10^{-98}	10^{97}
sub $= \tau\alpha a_{ijk}$	σ_{ij}^2	6460	7128	0	1	1
a_k, sub $= \tau\alpha a_{ijk}$	σ_{ij}^2	6541	7209	81	10^{-18}	10^{17}
a_k,τa_{ik},sub $= \tau\alpha a_{ijk}$	σ_{ij}^2	6567	7243	115	10^{-26}	10^{25}
a_k,αa_{jk},sub $= \tau\alpha a_{ijk}$	σ_{ij}^2	6537	7213	85	10^{-19}	10^{18}
$a_k, \tau a_{ik}, \alpha a_{jk}$, sub $= \tau\alpha a_{ijk}$	σ_{ij}^2	6583	7264	135	10^{-30}	10^{29}

[a] σ^2, homoscedastic assumption; σ_i^2, heteroscedasticity for matrix; σ_j^2, heteroscedasticity for method; and σ_{ij}^2, heteroscedasticity for feed-method groups.

[b] ℓ^* is the logarithm of the pseudo-likelihood; $AICc_r$ is the corrected Akaike information criterion; Δ_r is the Akaike difference; w_r is the model probability; and ER_r is the evidence ratio computed for each one of the r-th model combination evaluated.

[c] sub $= \tau\alpha a_{ijk}$ means that the subject chosen is the feed-matrix-analyst interaction.

Table 2. Least squares means, standard deviations (± SD), and 99% confidence intervals (lower and upper limits) for dry matter (g/kg as is) and major chemical constituents (g/kg DM) of the materials represented feed matrixes

Matrix no.	DM	OM	Ash	CF	CP	ADFom	Lignin (sa)
1	874 ± 0.5	989 ± 0.7	11 ± 0.7	42 ± 1.3	75 ± 3.7	28 ± 2.7	4 ± 1.0
	(873, 875)	(988, 990)	(10, 12)	(40, 45)	(68, 83)	(23, 34)	(2, 7)
2	225 ± 0.6	965 ± 1.2	35 ± 1.2	33 ± 1.2	327 ± 6.6	211 ± 6.7	75 ± 4.5
	(223, 226)	(963, 968)	(32, 37)	(30, 35)	(317, 338)	(198, 225)	(66, 84)
3	908 ± 0.4	950 ± 1.4	50 ± 1.4	230 ± 2.7	392 ± 6.9	80 ± 4.5	4 ± 1.1
	(907, 908)	(947, 953)	(47, 53)	(224, 235)	(378, 406)	(72, 90)	(2, 7)
4	894 ± 0.5	932 ± 1.6	68 ± 1.6	6 ± 0.5	451 ± 7.0	223 ± 6.9	74 ± 4.5
	(893, 895)	(929, 935)	(65, 71)	(5, 7)	(440, 462)	(209, 237)	(66, 84)
5	306 ± 0.7	937 ± 1.6	63 ± 1.6	19 ± 0.9	86 ± 4.0	276 ± 7.4	33 ± 3.1
	(305, 308)	(934, 940)	(60, 67)	(17, 21)	(79, 95)	(261, 291)	(27, 39)
6	889 ± 0.5	934 ± 1.6	66 ± 1.6	10 ± 0.6	96 ± 4.2	392 ± 8.0	52 ± 3.8
	(888, 890)	(931, 937)	(63, 69)	(8, 11)	(88, 105)	(376, 408)	(47, 59)
7	883 ± 0.5	898 ± 1.9	102 ± 1.9	15 ± 0.8	196 ± 5.6	398 ± 8.1	93 ± 5.0
	(882, 884)	(894, 902)	(98, 106)	(13, 17)	(185, 207)	(382, 414)	(85, 101)
8	897 ± 0.5	938± 1.5	62 ± 1.5	19 ± 0.9	58 ± 3.3	191 ± 6.5	18 ± 2.3
	(896, 898)	(935, 940)	(60, 65)	(17, 20)	(52, 65)	(179, 205)	(14, 22)
9	880 ± 0.5	932 ± 1.6	68 ± 1.6	14 ± 0.8	479 ± 7.0	91 ± 4.7	6 ± 1.3
	(879, 881)	(929, 935)	(65, 71)	(12, 15)	(465, 493)	(82, 101)	(4, 9)
10	886 ± 0.5	989 ± 0.7	11 ± 0.7	11 ± 0.7	77 ± 3.8	42 ± 3.3	5 ± 1.2
	(886, 887)	(988, 991)	(10, 12)	(10, 13)	(70, 85)	(36, 49)	(3, 8)
11	865 ± 0.5	947 ± 1.4	53 ± 1.4	37 ± 1.2	167 ± 5.3	121 ± 5.4	37 ± 3.3
	(864, 866)	(944, 949)	(51, 56)	(34, 39)	(157, 178)	(111, 132)	(31, 44)
12	155 ± 0.6	898 ± 1.9	102 ± 1.9	18 ± 0.9	131 ± 4.8	372 ± 8.0	34 ± 3.1
	(154, 156)	(894, 902)	(98, 106)	(16, 20)	(118, 145)	(356, 388)	(28, 41)
13	897 ± 0.5	949 ± 1.4	51 ± 1.4	29 ± 1.1	222 ± 5.9	125 ± 5.4	11 ± 1.8
	(896, 898)	(946, 952)	(48, 54)	(27, 32)	(210, 234)	(114, 136)	(8, 15)
14	899 ± 0.5	979 ± 0.9	22 ± 0.9	3 ± 0.4	20 ± 2.0	463 ± 8.2	41 ± 3.4
	(898, 900)	(977, 980)	(20, 23)	(3, 4)	(16, 24)	(447, 480)	(35, 48)

Table 2. (Continued)

Matrix no.	DM	OM	Ash	CF	CP	ADFom	Lignin (sa)
15	249 ± 0.7 (248, 256)	961 ± 1.2 (958, 963)	40 ± 1.2 (37, 42)	13 ± 0.7 (12, 15)	35 ± 2.6 (30, 41)	298 ± 7.5 (283, 313)	43 ± 3.5 (37, 51)
16	954 ± 0.3 (953, 955)	933 ± 1.6 (930, 937)	67 ± 1.6 (64, 70)	145 ± 2.3 (138, 151)	174 ± 5.4 (164, 185)	2 ± 0.8 (1, 4)	3 ± 1.0 (2, 6)
17	886 ± 0.5 (885, 887)	952 ± 1.4 (950, 955)	48 ± 1.4 (45, 51)	16 ± 0.8 (14, 17)	118 ± 4.5 (109, 127)	506 ± 8.2 (489, 522)	2 ± 2.6 (19, 30)
18	926 ± 0.4 (926, 927)	617 ± 3.1 (611, 623)	383 ± 3.1 (377, 389)	113 ± 2.1 (109, 118)	473 ± 7.0 (459, 487)	10 ± 1.6 (7, 14)	10 ± 1.7 (7, 14)
19	914 ± 0.4 (913, 915)	997 ± 0.4 (996, 997)	4 ± 0.4 (3, 4)	1 ± 0.2 (1, 1)	25 ± 2.2 (21, 29)	794 ± 6.7 (780, 807)	272 ± 7.6 (257, 287)
20	900 ± 0.5 (899, 901)	935 ± 1.6 (932, 937)	65 ± 1.6 (63, 68)	8 ± 0.6 (7, 9)	93 ± 4.1 (85, 102)	465 ± 8.2 (449, 482)	60 ± 4.1 (52, 69)
P-Value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

1, Corn, yellow, grounded; 2, Barley, wet brewers grain; 3, Soybean, seeds, whole; 4, Cottonseed meal solvent; 5, Corn silage; 6, Tyfton 85, 45-days harvested hay; 7, Alfalfa, 28-days harvested hay; 8, citrus, pulp dried; 9, Soybean meal, solvent; 10, Sorghum, grain; 11, Wheat middlings; 12, Elephant-grass, harvested at 170 cm height; 13, Corn, gluten feed, dried; 14, Corn husks; 15, Sugar cane; 16, Powdered milk; 17, Soybean hulls; 18, Meat and bone meal; 19, Sawdust; and 20, Ryegrass, late cut hay.
DM, dry matter; OM, organic matter; CF, crude fat; CP, crude protein; ADFom, ADF exclusive of residual ash; Lignin (sa), lignin determined by solubilization with 72% sulphuric acid over the acid detergent residue.

Table 3. Least squares means and standard deviations ($\pm$ SD$_{r_{ij}}$) for aNDFom of each material × method interaction estimated by restricted maximum likelihood assuming normally distributed errors, and *P*-values regarding interaction effect slices

Matrix	Method						*P*-value
no.	1	2	3	4	5	6	
1	106 $\pm$ 5.5	98 $\pm$ 8.7*	101 $\pm$ 12.0*	81 $\pm$ 6.0*	230 $\pm$ 109.8*	149 $\pm$ 23.0*	<0.001
2	448 $\pm$ 10.8	450 $\pm$ 5.6	444 $\pm$ 8.0	456 $\pm$ 9.5	491 $\pm$ 28.5*	474 $\pm$ 18.0	0.009
3	126 $\pm$ 38.3*	152 $\pm$ 18.4*	105 $\pm$ 6.7*	137 $\pm$ 8.7*	256 $\pm$ 93.9*	162 $\pm$ 28.0*	<0.001
4	279 $\pm$ 16.4*	294 $\pm$ 19.5*	285 $\pm$ 12.4	272 $\pm$ 23.3*	333 $\pm$ 45.6*	299 $\pm$ 27.5*	0.081
5	468 $\pm$ 13.7	487 $\pm$ 10.9	467 $\pm$ 12.1	451 $\pm$ 11.9	463 $\pm$ 10.4	469 $\pm$ 17.7	0.002
6	769 $\pm$ 6.6	788 $\pm$ 33.0*	786 $\pm$ 12.1	757 $\pm$ 20.9	764 $\pm$ 9.8	778 $\pm$ 18.8	0.027
7	513 $\pm$ 14.1	506 $\pm$ 18.8	515 $\pm$ 15.0	492 $\pm$ 15.9	521 $\pm$ 18.3	516 $\pm$ 18.4	0.053
8	194 $\pm$ 0.7	193 $\pm$ 2.0	196 $\pm$ 5.2	194 $\pm$ 4.0	218 $\pm$ 13.4	212 $\pm$ 10.8*	0.005
9	134 $\pm$ 7.6*	142 $\pm$ 7.1	145 $\pm$ 10.0*	135 $\pm$ 9.1*	212 $\pm$ 65.7*	159 $\pm$ 24.3*	0.037
10	99 $\pm$ 5.7*	93 $\pm$ 19.5*	102 $\pm$ 12.9*	81 $\pm$ 9.6*	262 $\pm$ 111.7*	208 $\pm$ 103.9*	<0.001
11	404 $\pm$ 8.8	409 $\pm$ 18.3	405 $\pm$ 9.8	390 $\pm$ 7.5	423 $\pm$ 47.4*	405 $\pm$ 12.5	0.031
12	649 $\pm$ 8.3	652 $\pm$ 4.0	655 $\pm$ 6.8	636 $\pm$ 10.5	646 $\pm$ 12.2	645 $\pm$ 13.8	0.018
13	465 $\pm$ 7.5	463 $\pm$ 8.7	463 $\pm$ 14.7	442 $\pm$ 5.0	473 $\pm$ 20.9	446 $\pm$ 13.5	<0.001
14	898 $\pm$ 12.6	898 $\pm$ 8.0	908 $\pm$ 17.1	879 $\pm$ 33.6	878 $\pm$ 11.8	889 $\pm$ 14.6	0.037
15	498 $\pm$ 5.0	494 $\pm$ 3.6	498 $\pm$ 5.8	462 $\pm$ 8.1	490 $\pm$ 9.3	485 $\pm$ 9.8	<0.001
16	8 $\pm$ 6.0*	5 $\pm$ 2.5*	1 $\pm$ 1.6*	0 $\pm$ 2.1#	48 $\pm$ 46.5*	6 $\pm$ 8.1*	0.009
17	688 $\pm$ 11.1	681 $\pm$ 5.3	687 $\pm$ 8.9	664 $\pm$ 8.9	666.$\pm$ 12.0	721 $\pm$ 22.5	<0.001
18	252 $\pm$ 51.3*	218 $\pm$ 11.5*	205 $\pm$ 7.1	190 $\pm$ 11.5*	274 $\pm$ 55.4*	203 $\pm$ 8.0	0.008
19	876 $\pm$ 6.9	876 $\pm$ 3.0	891 $\pm$ 7.4	841 $\pm$ 9.0	848 $\pm$ 13.6	878 $\pm$ 14.2	<0.001
20	775 $\pm$ 4.1	782 $\pm$ 5.4	790 $\pm$ 10.5	764 $\pm$ 6.3	769 $\pm$ 9.7	775 $\pm$ 12.5	0.001
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	

Materials: 1, Corn, yellow, grounded; 2, Barley, wet brewers grain; 3, Soybean, seeds, whole; 4, Cottonseed meal solvent; 5, Corn silage; 6, Tyfton 85, 45-days harvested hay; 7, Alfalfa, 28-days harvested hay; 8, citrus, pulp dried; 9, Soybean meal, solvent; 10, Sorghum, grain; 11, Wheat middlings; 12, Elephant-grass, harvested at 170 cm height; 13, Corn, gluten feed, dried; 14, Corn husks; 15, Sugar cane; 16, Powdered milk; 17, Soybean hulls; 18, Meat and bone meal; 19, Sawdust; and 20, Ryegrass, late cut hay. Methods: 1, Fibertec, AOAC 2002.04; 2, Reflux in beakers, AOAC 2002.04; 3, Reflux in beakers with spouts (modified AOAC 2002.04); 4, Ankom, filter bags; 5, Tecnal, nonwoven tissue filter bags; and 6, Micro NDF (autoclave). * indicates RSD$_{r_{ij}}$ < RSD$_{R}$ (see section 2.4.3) and # indicates that RSD$_{R}$ cannot be computed because of a zeroed concentration estimate.

Table 5. Measures of the adequacy of the different methods (number) with regard to the AOAC 2002.04 Fibertec (1) and reflux in beakers (2) as reference fiber values for all materials represented feed matrixes

Comparisons	$\bar{x}$	$\hat{\sigma}$	$\tilde{x}$	R^2	MSEP	MB	MEF	CD	C_b	$\hat{\rho}_c$	$H_0^{(1)}$	$H_0^{(2)}$
Fibertec (1)	433	277.5	457	–	–	–	–	–	–	–	–	–
(1) × (3)	433	284.9	453	0.998	193.7	0	0.997	0.949	1.000	0.999	0.046	0.060
(1) × (4)	416	274.4	447	0.997	522.1	17	0.993	1.019	0.998	0.996	0.001	0.002
(1) × (5)	463	237.4	468	0.980	3741.8	-31	0.949	1.343	0.981	0.971	<0.001	<0.001
(1) × (6)	444	267.8	458	0.988	1068.5	-12	0.985	1.072	0.998	0.992	0.165	0.195
Reflux (2)	434	279.3	456	–	–	–	–	–	–	–	–	–
(2) × (3)	433	284.9	453	0.998	179.5	2	0.984	0.961	1.000	0.999	0.927	0.934
(2) × (4)	416	274.4	447	0.999	437.7	18	0.994	1.032	0.998	0.997	<0.001	<0.001
(2) × (5)	463	237.4	468	0.981	3710.2	-29	0.950	1.363	0.981	0.971	<0.001	<0.001
(2) × (6)	444	267.8	458	0.989	1006.6	-10	0.986	1.086	0.998	0.993	0.146	0.174
(2) × (1)	–	–	–	0.998	163.8	2	0.998	1.013	1.000	0.999	0.776	0.796

$\bar{x}$, mean; $\hat{\sigma}$, standard deviation; $\tilde{x}$, median; R^2, coefficient of determination of the linear regression; MSEP, mean squared error of prediction; MB, mean bias; MEF, method efficiency factor; CD coefficient of determination of the method; C_b, method accuracy; $\hat{\rho}_c$, concordance correlation coefficient; $H_0^{(1)}$: $[\theta_0 \quad \theta_1] = [0 \quad 1]$ (Mayer et al., 1994), and $H_0^{(2)}$: $[\theta_0 \quad \theta_1] = [0 \quad 1]$ (Dent and Blackie, 1979).
Methods: 1, Fibertec, AOAC 2002.04; 2, Reflux in beakers, AOAC 2002.04; 3, Reflux in beakers with spouts (modified AOAC 2002.04); 4, Pressurized filter bags (ANKOM); 5, Non-pressurized filter bags (TECNAL); and 6, Micro-NDF (autoclave).

Table 6. Least squares means and standard deviations (± SD) for aNDFom (g/kg DM) of each material × method interaction estimated by restricted maximum likelihood assuming aNDFom/1000 as a beta distributed variable, and *P*-values regarding interaction effect slices

Matrix	Method						*P*-value
no.	1	2	3	4	5	6	
1	107 ± 5.5	98 ± 8.7	101 ± 12.0	81 ± 6.1	230 ± 109.8	149 ± 23.0	<0.001
2	448 ± 10.8	450 ± 5.7	444 ± 7.9	456 ± 9.5	491 ± 28.5	474 ± 18.0	0.009
3	126 ± 38.3	152 ± 18.4	105 ± 6.63	137 ± 8.7	256 ± 93.9	162 ± 28.0	<0.001
4	279 ± 16.4	294 ± 19.4	285.4 ± 12.5	272 ± 23.4	333 ± 45.6	299 ± 27.5	0.073
5	468 ± 13.7	487 ± 10.9	467 ± 12.1	451 ± 11.9	463 ± 10.4	470 ± 17.7	0.002
6	769 ± 6.6	788.4 ± 33.0	786 ± 12.0	757 ± 20.9	764 ± 9.8	778 ± 18.8	0.029
7	513 ± 14.1	506 ± 18.8	515 ± 15.0	492 ± 15.9	521 ± 18.3	516 ± 18.4	0.053
8	194 ± 1.0	193 ± 2.0	196 ± 5.2	194 ± 4.0	218 ± 13.4	212 ± 10.8	0.004
9	134 ± 7.6	142 ± 7.1	145 ± 10.0	135 ± 9.1	212 ± 65.7	159 ± 24.3	0.023
10	99 ± 5.7	93 ± 19.5	102 ± 12.9	81 ± 9.6	262 ± 111.7	208 ± 103.9	<0.001
11	404 ± 8.8	409 ± 18.3	405 ± 9.8	390 ± 7.6	423 ± 47.4	405 ± 12.5	0.031
12	649 ± 8.3	652 ± 4.0	655 ± 6.8	636 ± 10.5	646 ± 12.3	645 ± 13.9	0.017
13	465 ± 7.5	463 ± 8.7	463 ± 14.7	442 ± 5.0	473 ± 20.9	446 ± 13.5	<0.001
14	898 ± 12.7	898 ± 8.0	908 ± 17.1	879 ± 33.7	878 ± 11.8	889 ± 14.6	0.034
15	498 ± 5.0	494 ± 3.6	498 ± 5.7	462 ± 8.1	490 ± 9.3	485 ± 9.8	<0.001
17	688 ± 11.1	681 ± 5.3	687 ± 8.9	665 ± 8.9	666 ± 12.0	721 ± 22.5	0.001
18	252 ± 51.3	218 ± 11.5	205 ± 7.1	190 ± 11.5	274 ± 55.4	203 ± 8.0	0.006
19	876 ± 6.9	876 ± 3.0	891 ± 7.4	841 ± 9.0	848 ± 13.6	878 ± 14.2	<0.001
20	775 ± 4.0	783 ± 5.4	790 ± 10.5	764 ± 6.3	769 ± 9.7	775 ± 12.5	0.001
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	

Materials: 1, Corn, yellow, grounded; 2, Barley, wet brewers grain; 3, Soybean, seeds, whole; 4, Cottonseed meal solvent; 5, Corn silage; 6, Tyfton 85, 45-days harvested hay; 7, Alfalfa, 28-days harvested hay; 8, citrus, pulp dried; 9, Soybean meal, solvent; 10, Sorghum, grain; 11, Wheat middlings; 12, Elephant-grass, harvested at 170 cm height; 13, Corn, gluten feed, dried; 14, Corn husks; 15, Sugar cane; 17, Soybean hulls; 18, Meat and bone meal; 19, Sawdust; and 20, Ryegrass, late cut hay.
Methods: 1, Fibertec, AOAC 2002.04; 2, Reflux in beakers, AOAC 2002.04; 3, Reflux in beakers with spouts (modified AOAC 2002.04); 4, Pressurized filter bags (ANKOM); 5, Non-pressurized filter bags; and 6, Micro NDF (autoclave).

Table 7. Significant contrasts between aNDFom methods (adjusted *P*-values) estimated by restricted maximum likelihood assuming aNDFom/1000 as a beta distributed variable

Matrix no.	Contrasts between methods within materials
1	1-4 (<0.001), 1-5 (0.005), 2-4 (0.008), 2-5 (0.001), 2-6 (0.006), 3-5 (0.002) , 4-5 (<0.001), 4-6 (0.001)
3	2-3 (0.001), 3-4 (<0.001), 3-5 (<0.001), 4-5 (0.002)
5	2-4 (0.001)
10	1-4 (0.002), 1-5 (<0.001), 1-6 (0.002), 2-5 (<0.001), 2-6 (0.002), 3-5 (<0.001), 3-6 (0.004), 4-5 (<0.001), 4-6 (<0.001)
12	2-4 (0.004)
13	1-4 (<0.001), 2-4 (<0.001)
15	1-4 (<0.001), 2-4 (<0.001), 3-4 (<0.001) , 4-5 (0.002), 4-6 (0.004)
17	2-4 (0.004), 3-4 (0.007)
18	2-4 (0.008)
19	1-4 (<0.001), 2-4 (<0.001), 3-4 (<0.001), 3-5 (0.001)
20	1-4 (0.007), 2-4 (0.001)

Materials: 1, Corn, yellow, grounded; 3, Soybean, seeds, whole; 5, Corn silage; 10, Sorghum, grain; 12, Elephant-grass, harvested at 170 cm height; 13, Corn, gluten feed, dried; 15, Sugar cane; 17, Soybean hulls; 18, Meat and bone meal; 19, Sawdust; and 20, Ryegrass, late cut hay.

Methods: 1, Fibertec, AOAC 2002.04; 2, Reflux in beakers, AOAC 2002.04; 3, Reflux in beakers with spouts (modified AOAC 2002.04); 4, Pressurized filter bags (ANKOM); 5, Non-pressurized filter bags; and 6, Micro NDF (autoclave).

Table 8. Least squares means and standard deviations (± SD) for Neutral detergent solubles (g/kg DM) of each material × method interaction estimated by restricted maximum likelihood assuming normally distributed errors, and *P*-values regarding interaction effect slices

Material	Method						*P*-value
	1	2	3	4	5	6	
1	894 ± 5.5	902 ± 8.7	899 ± 12.0	919 ± 6.1	768 ± 109.8	851 ± 23.0	<0.001
2	552 ± 10.8	550 ± 5.6	556 ± 8.0	544 ± 9.5	509 ± 28.5	526 ± 18.0	0.009
3	874 ± 38.3	848 ± 18.4	895 ± 6.7	863 ± 8.7	744 ± 93.9	838 ± 28.0	<0.001
4	721 ± 16.4	706 ± 19.5	715 ± 12.4	728 ± 23.3	667 ± 45.6	701 ± 27.5	0.081
5	532 ± 13.7	513 ± 10.9	533 ± 12.1	549 ± 11.9	537 ± 10.4	531 ± 17.7	0.002
6	231 ± 6.6	212 ± 33.0	214 ± 12.1	243 ± 20.9	236 ± 9.8	222 ± 18.8	0.027
7	487 ± 14.1	494 ± 18.8	485 ± 15.0	508 ± 15.9	479 ± 18.3	484 ± 18.4	0.053
8	806 ± 0.7	807 ± 2.0	804 ± 5.2	806 ± 4.0	782 ± 13.4	788 ± 10.8	0.005
9	866 ± 7.6	858 ± 7.1	855 ± 10.0	865 ± 9.1	788 ± 65.7	841 ± 24.3	0.037
10	901 ± 5.7	907 ± 19.5	899 ± 12.9	920 ± 9.6	738 ± 111.7	792 ± 103.9	<0.001
11	596 ± 8.8	591 ± 18.3	595 ± 9.8	610 ± 7.5	577 ± 47.4	595 ± 12.5	0.031
12	351 ± 8.3	348 ± 4.0	345 ± 6.8	364 ± 10.5	354 ± 12.2	356 ± 13.8	0.018
13	535 ± 7.5	537 ± 8.7	537 ± 14.7	558 ± 5.0	527 ± 20.9	554 ± 13.5	<0.001
14	102 ± 12.6	102 ± 8.0	92 ± 17.1	121 ± 33.6	123 ± 11.8	111 ± 14.6	0.037
15	502 ± 5.0	506 ± 3.6	502 ± 5.8	538 ± 8.1	510 ± 9.3	515 ± 9.8	<0.001
16	992 ± 6.0	995 ± 2.5	999 ± 1.7	1000 ± 2.1	953 ± 46.5	994 ± 8.1	0.009
17	312 ± 11.2	320 ± 5.3	313 ± 8.9	336 ± 8.9	334 ± 12.0	279 ± 22.5	<0.001
18	748 ± 51.3	783 ± 11.5	795 ± 7.1	810 ± 11.5	726 ± 55.4	797 ± 8.0	0.008
19	124 ± 7.0	124 ± 3.0	109 ± 7.4	159 ± 9.0	152 ± 13.6	122 ± 14.2	<0.001
20	225 ± 4.1	218 ± 5.4	210 ± 10.5	236 ± 6.4	231 ± 9.7	225 ± 12.5	0.001
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	

Materials: 1, Corn, yellow, grounded; 2, Barley, wet brewers grain; 3, Soybean, seeds, whole; 4, Cottonseed meal solvent; 5, Corn silage; 6, Tyfton 85, 45-days harvested hay; 7, Alfalfa, 28-days harvested hay; 8, citrus, pulp dried; 9, Soybean meal, solvent; 10, Sorghum, grain; 11, Wheat middlings; 12, Elephant-grass, harvested at 170 cm height; 13, Corn, gluten feed, dried; 14, Corn husks; 15, Sugar cane; 16, Powdered milk; 17, Soybean hulls; 18, Meat and bone meal; 19, Sawdust; and 20, Ryegrass, late cut hay.

Methods: 1, Fibertec, AOAC 2002.04; 2, Reflux in beakers, AOAC 2002.04; 3, Reflux in beakers with spouts (modified AOAC 2002.04); 4, Pressurized filter bags (ANKOM); 5, Non-pressurized filter bags; and 6, Micro NDF (autoclave).